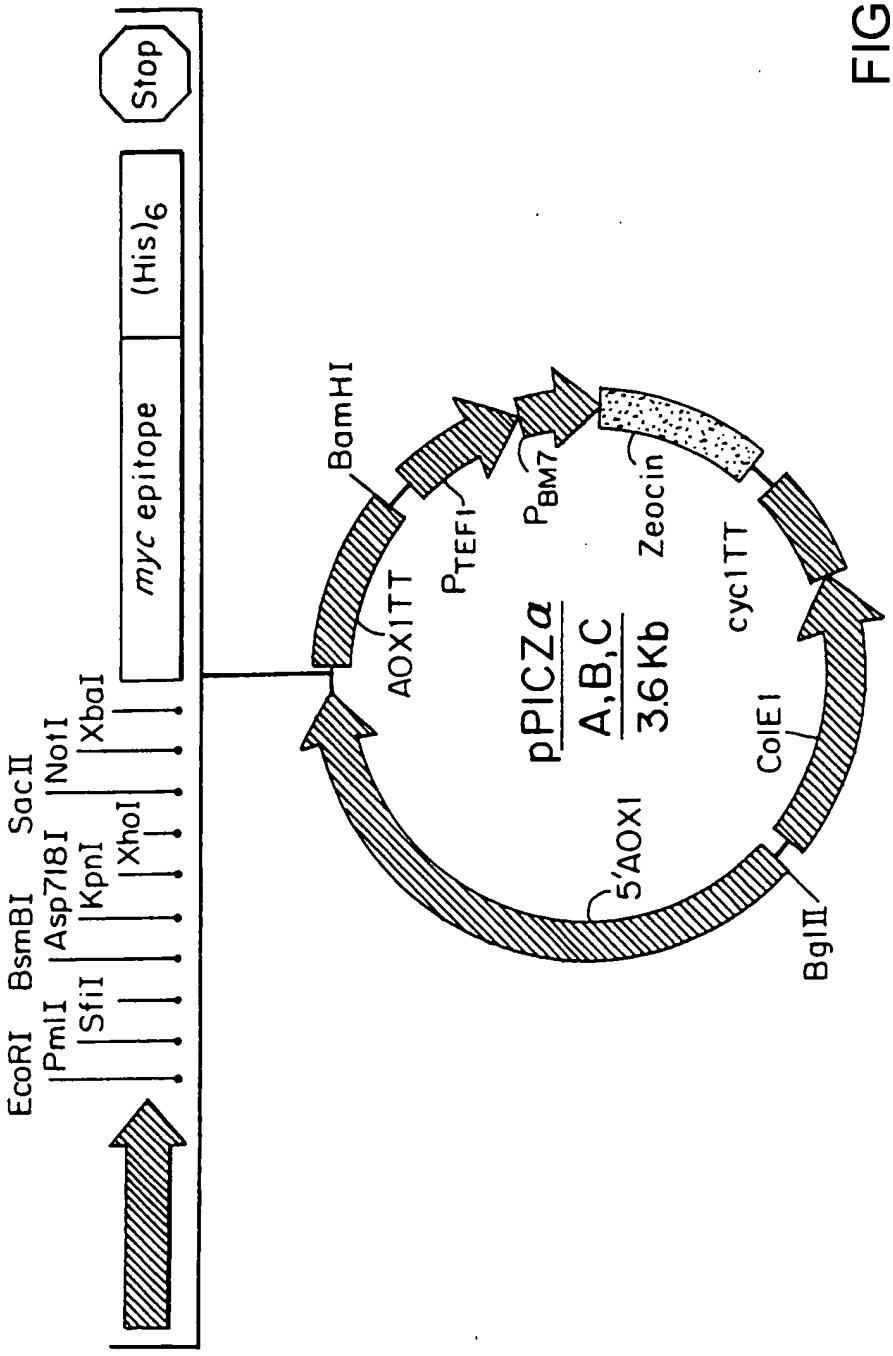
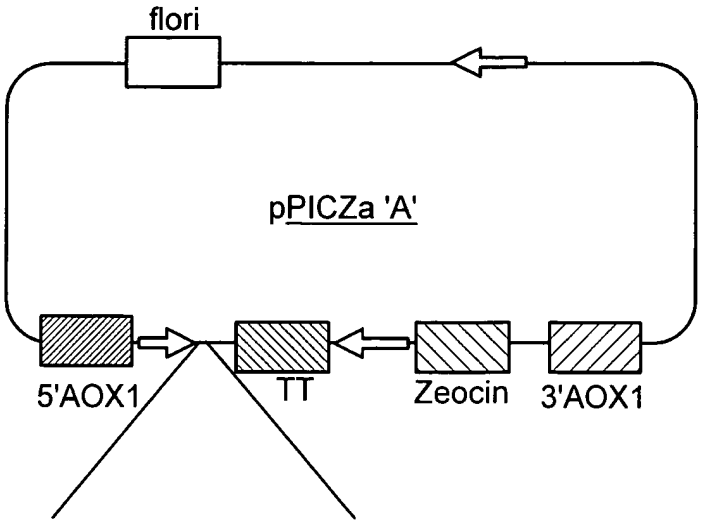


FIG. 1



MULTIPLE CLONING SITES
Eco RI, / Pml I / Sfi I / Bsm BI / Asp718I / Kpn I / Xho I/
Sac II / Not I / Xba I

↓

DIGEST THE VECTOR WITH EcoRI RESTRICTION ENZYME

↓

DENATURE AND ANNEAL TWO COMPLIMENTARY OLIGO WHICH WOULD INCORPORATE His. Tag MOTIF AND Nde I, Nhe I RESTRICTION SITES (His. Tag REGION WOULD BE HELPFUL TO SIMPLIFY PURIFICATION AND THE PRESENCE OF Nde I AND Nhe I RESTRICTION SITES FACILITATE SHUTTLING OF PCR FROM PROKARYOTIC EXPRESSION SYSTEM TO YEAST EXPRESSION WITHOUT GOING THROUGH PCR AMPLIFICATION PROCESS)

5' AAT TCC ATC ACC ATC ACC ATC ACC ATA TGG CTA GCA 3'
5' AAT TTG CTA GCC ATA TGG TGA TGG TGA TGG TGA TGG 3'

MODIFIED PICHIA EXPRESSION VECTOR (pPICZ. His. A)

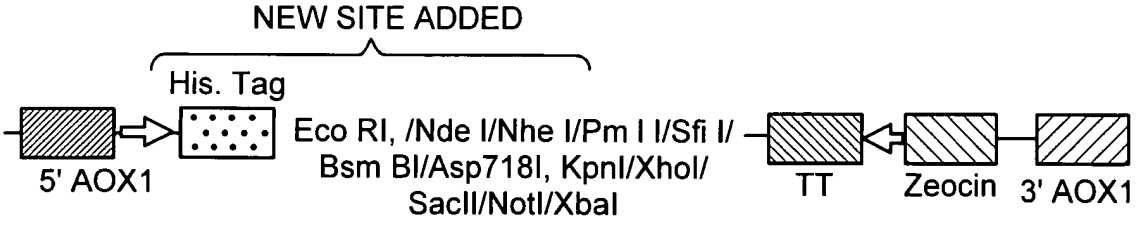


FIG. 2

FLOW CHART: CLONING OF ENDOSTATIN (MOUSE) INTO PICHIA EXPRESSION SYSTEM

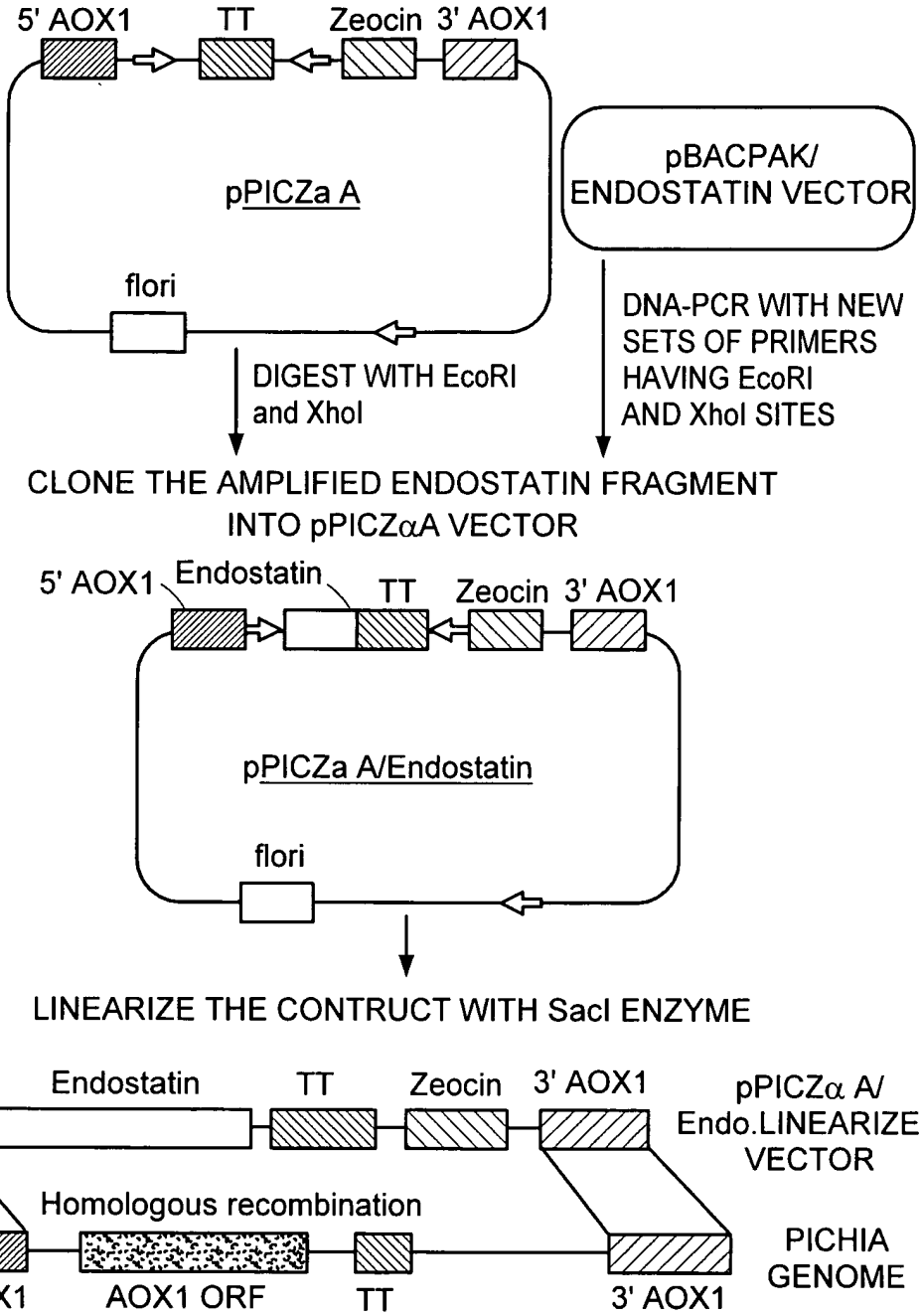


FIG. 3

FIG. 4A
FIG. 4B

FIG. 4

endo sequence from Collagen XVIII.
Sequence Range: 1-555
Nucleotide 1 = Start for Endostatin and fragments EM1 and EM2.
EM1 fragment ends at nucleotide 525, EM2 fragment ends at nucleotide 501.

```
      5     10    15     20     25    30     35     40    45
CAT ACT CAT CAG GAC TTT CAG CCA GTG CTC CAC CTG GTG GCA CTG AAC
GTA TGA GTA GTC CTG AAA GTC GGT CAC GAG GTG GAC CAC CGT GAC TTG

 50     55    60     65     70    75     80     85    90     95
ACC CCC CTG TCT GGA GGC ATG CGT GGT ATC CGT GGA GCA GAT TTC CAG
TGG GGG GAC AGA CCT CCG TAC GCA CCA TAG GCA CCT CGT CTA AAG GTC

  100   105    110    115   120    125    130   135    140
TGC TTC CAG CAA GCC CGA GCC GTG GGG CTG TCG GGC ACC TTC CGG GCT
ACG AAG GTC GTT CGG GCT CGG CAC CCC GAC AGC CCG TGG AAG GCC CGA

145   150    155    160   165    170    175   180    185    190
TTC CTG TCC TCT AGG CTG CAG GAT CTC TAT AGC ATC GTG CGC CGT GCT
AAG GAC AGG AGA TCC GAC GTC CTA GAG ATA TCG TAG CAC GCG GCA CGA

195    200    205   210    215    220   225    230    235   240
GAC CGG GGG TCT GTG CCC ATC GTC AAC CTG AAG GAC GAG GTG CTA TCT
CTG GCC CCC AGA CAC GGG TAG CAG TTG GAC TTC CTG CTC CAC GAT AGA
```

FIG. 4A

```
    245    250    255    260    265    270    275    280    285
 CCC AGC TGG GAC TCC CTG TTT TCT GGC TCC CAG GGT CAA CTG CAA CCC
 GGG TCG ACC CTG AGG GAC AAA AGA CCG AGG GTC CCA GTT GAC GTT GGG

290    295    300    305    310    315    320    325    330    335
 GGG GCC CGC ATC TTT TCT TTT GAC GGC AGA GAT GTC CTG AGA CAC CCA
 CCC CGG GCG TAG AAA AGA AAA CTG CCG TCT CTA CAG GAC TCT GTG GGT

   340    345    350    355    360    365    370    375    380
 GCC TGG CCG CAG AAG AGC GTA TGG CAC GGC TCG GAC CCC AGT GGG CGG
 CGG ACC GGC GTC TTC TCG CAT ACC GTG CCG AGC CTG GGG TCA CCC GCC

385   390    395    400    405    410    415    420    425    430
 AGG CTG ATG GAG AGT TAC TGT GAG ACA TGG CGA ACT GAA ACT ACT GGG
 TCC GAC TAC CTC TCA ATG ACA CTC TGT ACC GCT TGA CTT TGA TGA CCC

 435    440    445    450    455    460    465    470    475    480
 GCT ACA GGT CAG GCC TCC TCC CTG CTG TCA GGC AGG CTC CTG GAA CAG
 CGA TGT CCA GTC CGG AGG AGG GAC GAC AGT CCG TCC GAG GAC CTT GTC

    485    490    495    500    505    510    515    520    525
 AAA GCT GCG AGC TGC CAC AAC AGC TAC ATC GTC CTG TGC ATT GAG AAT
 TTT CGA CGC TCG ACG GTG TTG TCG ATG TAG CAG GAC ACG TAA CTC TTA

530    535    540    545    550    555
 AGC TTC ATG ACC TCT TTC TCC AAA TAG
 TCG AAG TAC TGG AGA AAG AGG TTT ATC
```

FIG. 4B

ENDOSTATIN

Construct Name	Primer Sequence	Cloning Sites	Vector	Protein Sequence
MOUSE	**ENDOSTATIN**			
pET17b/ his.mendo	5'-GGC ATA TGC ATA CTC ATC AGG- ACT TT-3' (up) (SEQ ID NO:4)	*NdeI* & *XhoI*	Prokaryotic expression, pET *(E. coli his.endo)*	MGHHHHHHHHHHSSGHIDDDDKHM-mendo (SEQ ID NO:14)
	5' AAC TCG AGC TAT TTG GAG AAA- GAG GT-3' (down) (SEQ ID NO:5)			
pET28a/ mendo	5'-GGC ATA TGC ATA CTC ATC AGG- ACT TT-3' (up) (SEQ ID NO:4)	*NdeI* & *NotI*	Prokaryotic expression, pET *(E. coli his.endo)*	MGSSHHHHHHSSGLVPRGSHM-mendo (SEQ ID NO:15)
	5'-AAG CGG CCG CCT ATT TGG AGA-AAG AGG T-3' (down) (SEQ ID NO:6)			
pET28a/ EM-1	5' TTC CAT ATG CAT ACT CAT CAG- GAC TTT CAG CCA-3' (up) (SEQ ID NO:7)		Prokaryotic expression, pET *(E. coli EM1)*	MGSSHHHHHHSSGLVPRGSHM-mendo (SEQ ID NO:15)
	5' TTA GCG GCC GCC TAC TCA ATG- CAC AGG ACG ATG TA-3' (down) (SEQ ID NO:8)			
pET28a/ EM-2	5' TTC CAT ATG CAT ACT CAT CAG- GAC TTT CAG CCA-3' (up) (SEQ ID NO:7)		Prokaryotic expression, pET *(E. coli EM2)*	MGSSHHHHHHSSGLVPRGSHM-mendo (SEQ ID NO:15)
	5' TTA GCG GCC GCC TAG TTG TGG- CAG CTC GCA GCT TTC TG-3' (down) (SEQ ID NO:9)			

FIG. 5A

Construct Name	Primer Sequence	Cloning Sites	Vector	Protein Sequence
pPICZαA/ mendo *(yeast mus endo)*	5' GGG AAT TCC ATA CTC ATC AGG- ACT TT-3' (up) (SEQ ID NO:10) 5' AAG CGG CCG CCT ATT TGG AGA- AAG AGG T-3' (down) (SEQ ID NO:6)	*Eco*RI & *Not*I	Eukaryotic expression, yeast/pPICZαA *(yeast mouse endostatin)*	EF-mendo (SEQ ID NO:16)
pPICZαA/ His.mendo	5' AAG AAT TCC ATC ATC ATC ATC- ATC ACA GCA GC-3' (up) (SEQ ID NO:11) 5' AAG CGG CCG CCT ATT TGG AGA- AAG AGG T-3' (down) (SEQ ID NO:6)	*Eco*RI & *Not*I	Eukaryotic expression, yeast/pPICZαA *(yeast mouse his.endostatin)*	EFMGHHHHHHHHHHSSGHIDDDD KHM-mendo (SEQ ID NO:17)
HUMAN	**ENDOSTATIN**			
pPICZαA/ Hendo	5' TTT GAA TTC GCC CAC AGC CAC- CGC GAC TTC CAG CCG GTG CTC- CAC-3' (up) (SEQ ID NO:12) 5' AAA AGC GGC CGC CTA CTT GGA- GGC AGT CAT GAA GCT GTT CTC- AAT-3' (down) (SEQ ID NO:13)	*Eco*RI & *Not*I	Eukaryotic expression, yeast/pPICZαA *(yeast human endostatin)*	EF-hendo (SEQ ID NO:18)

FIG. 5B

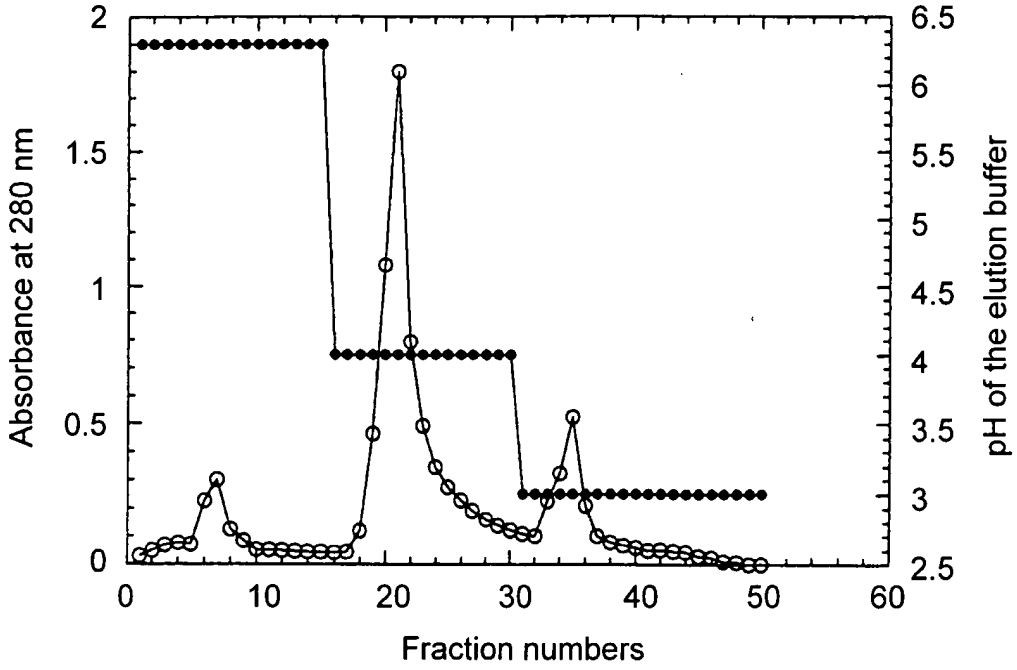


FIG. 6A

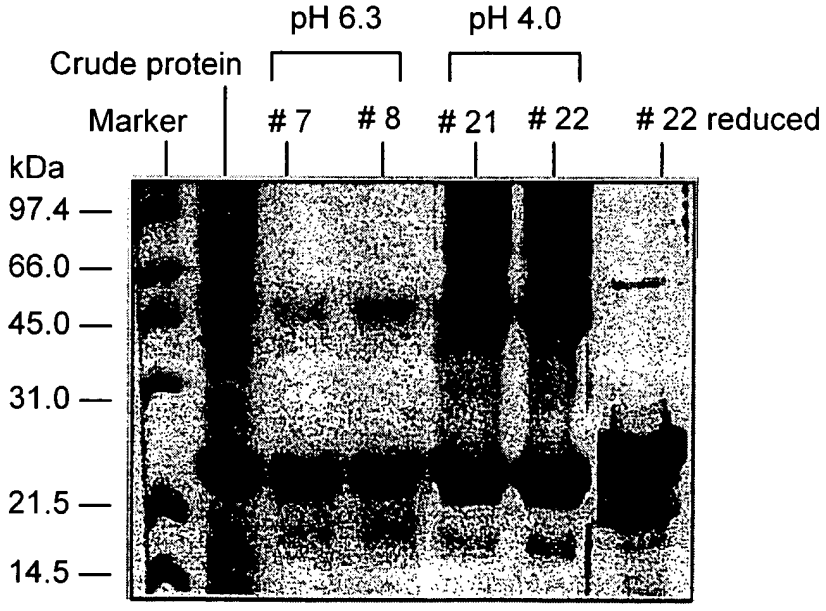


FIG. 6B

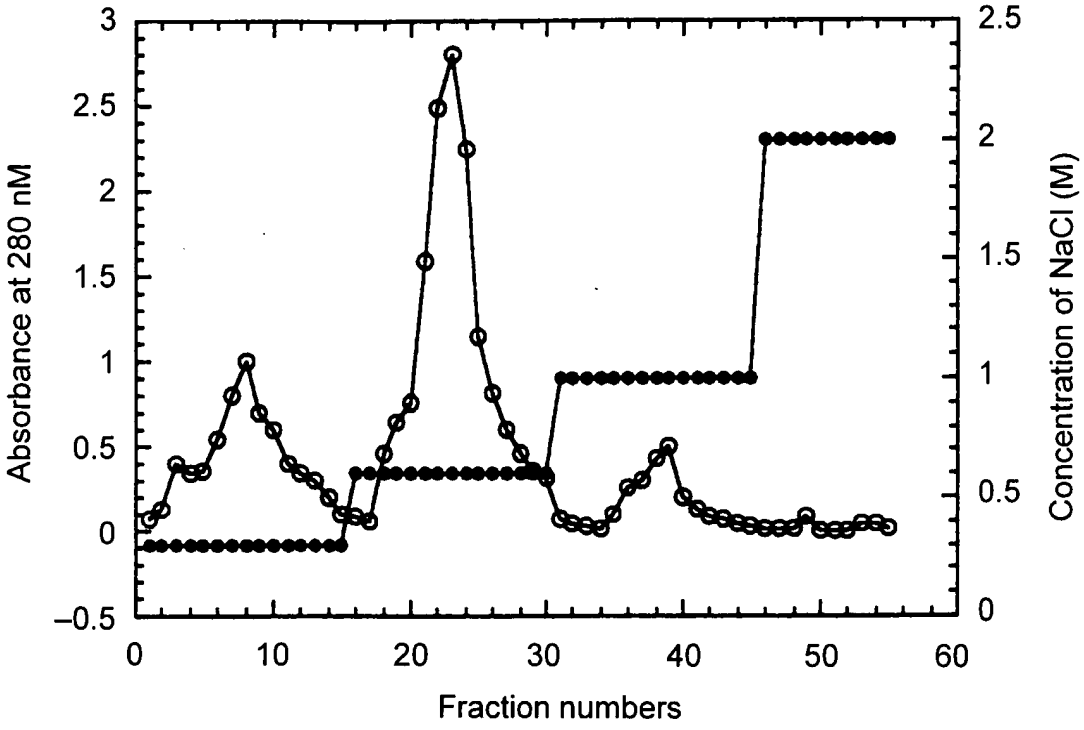


FIG. 7A

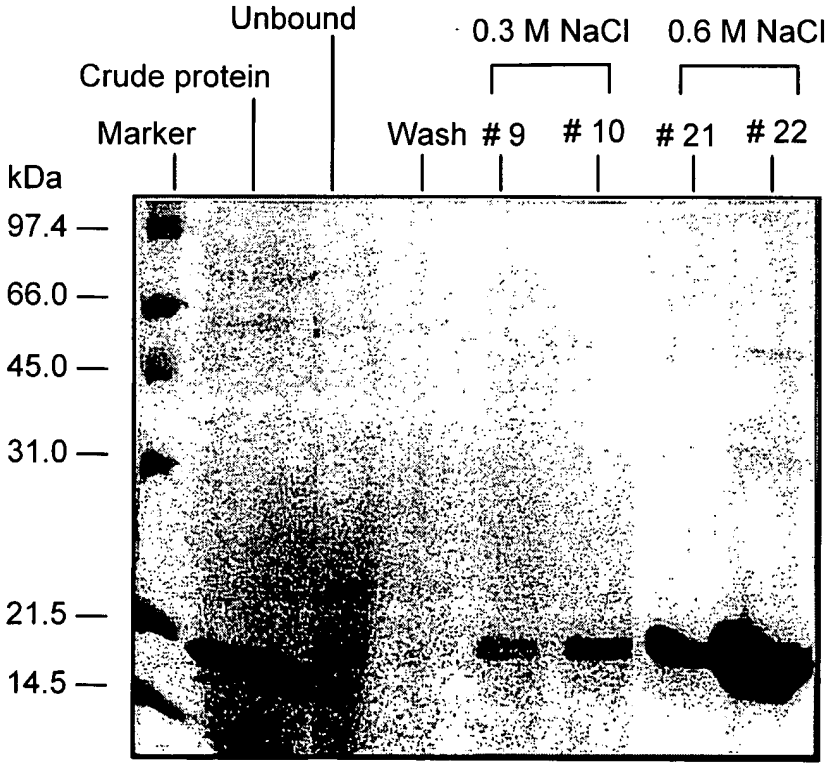


FIG. 7B

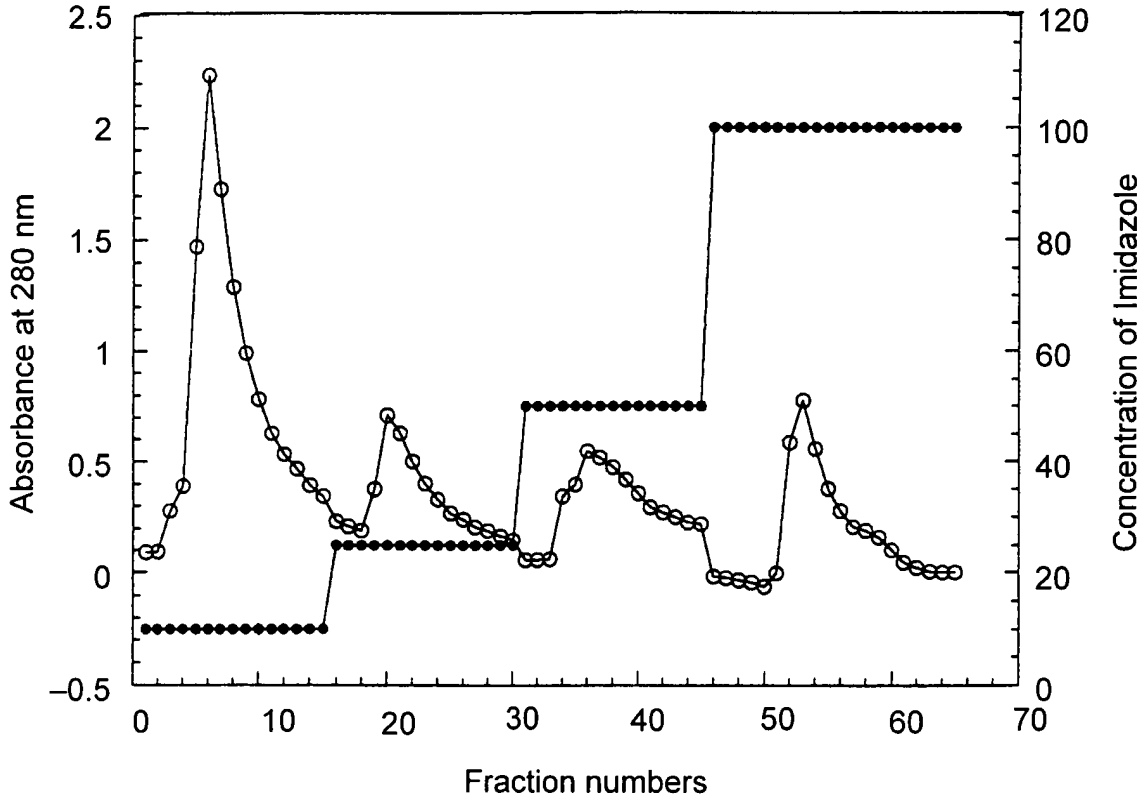


FIG. 8A

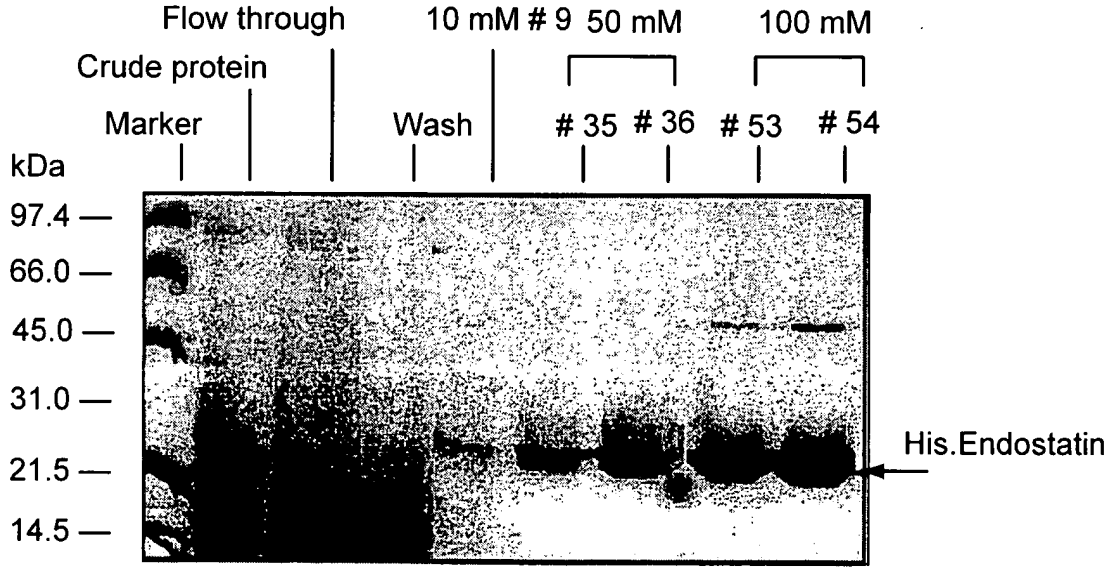


FIG. 8B

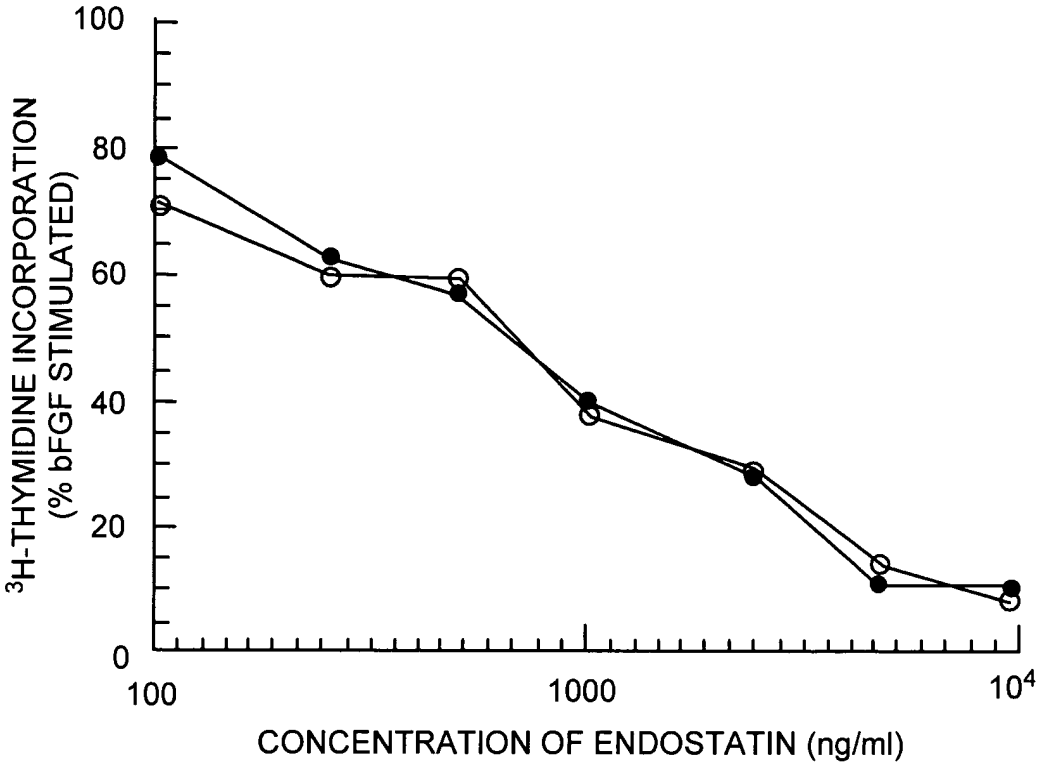


FIG. 9

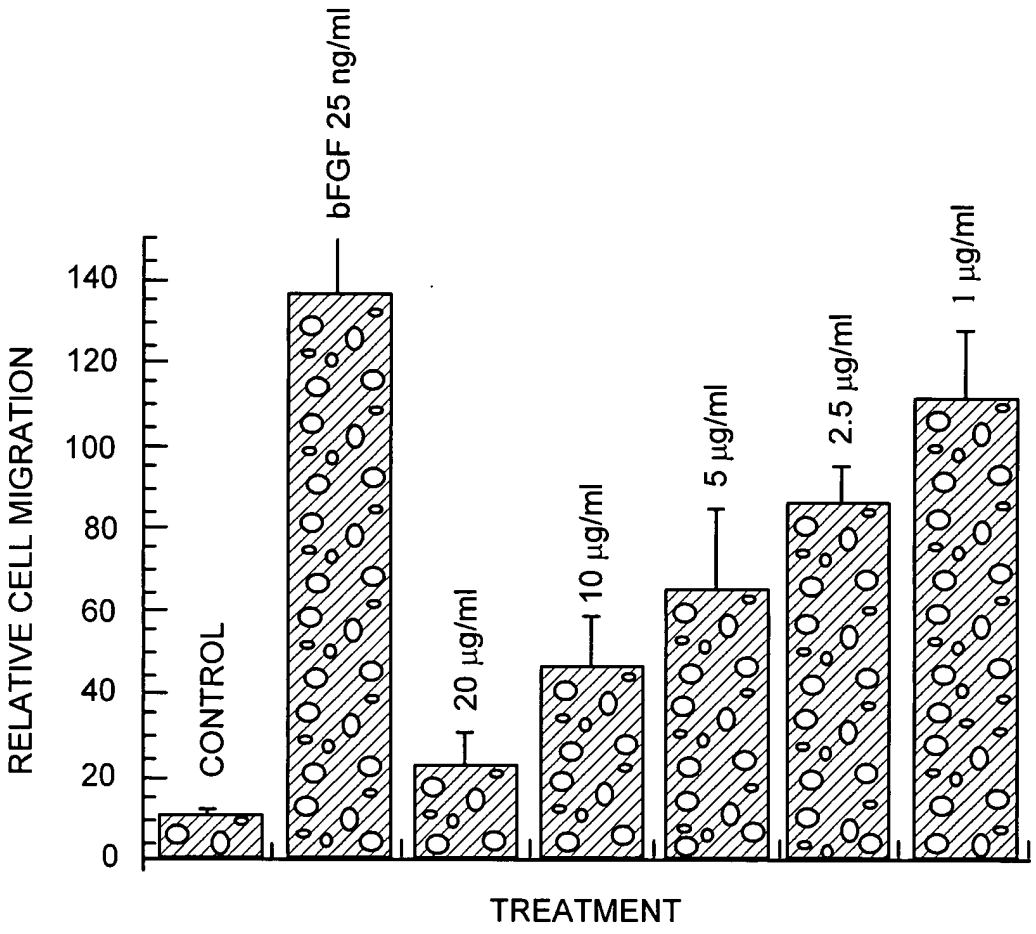


FIG. 10

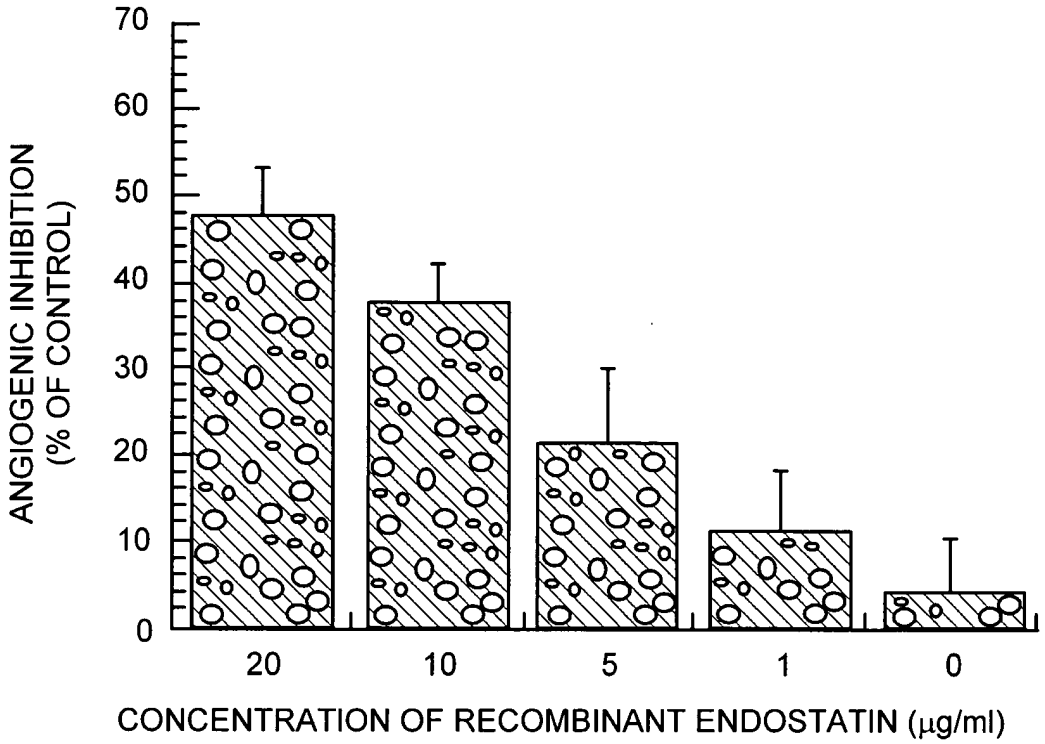


FIG. 11A

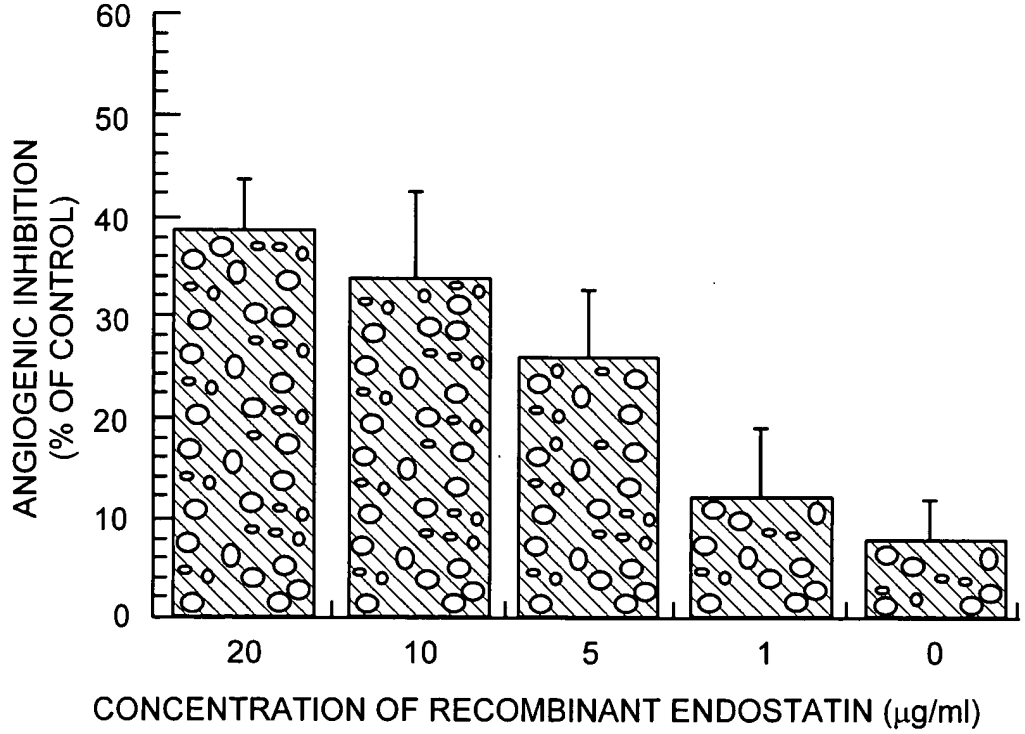


FIG. 11B

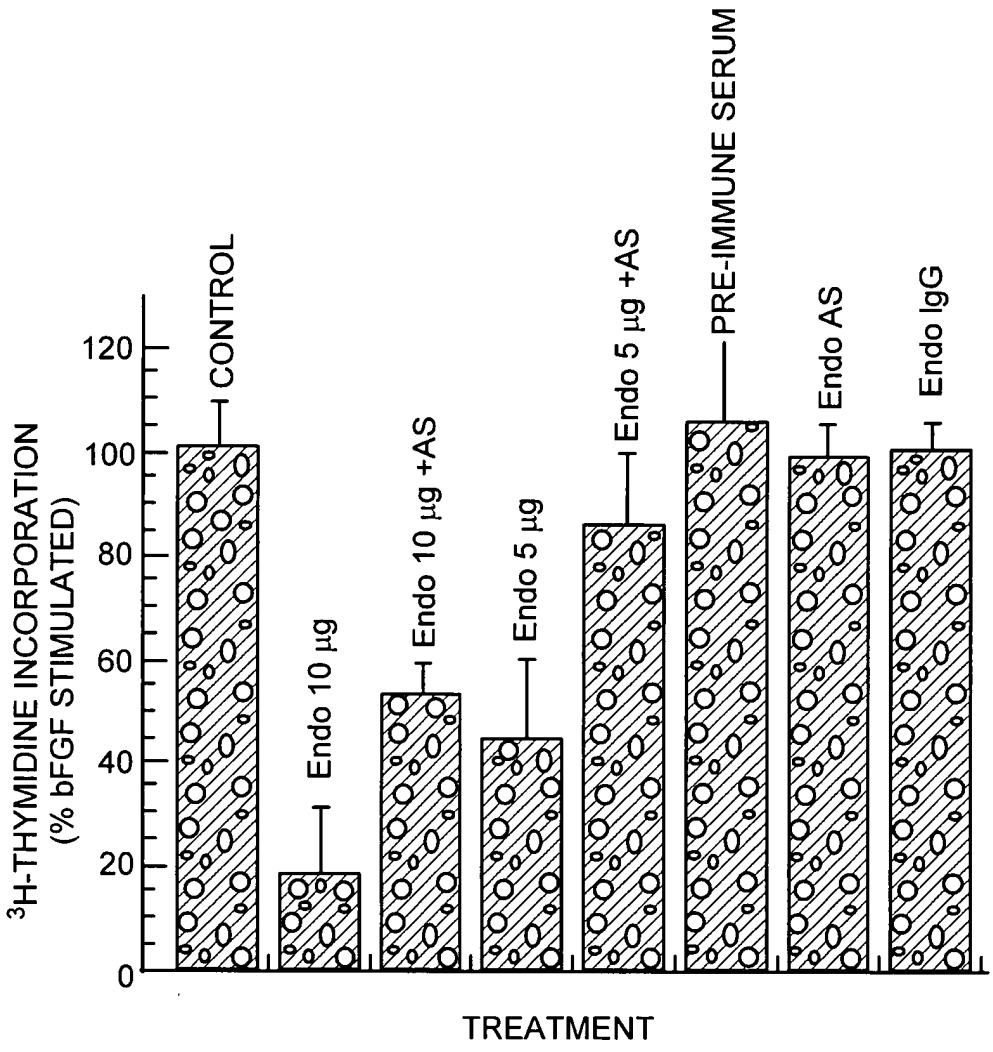


FIG. 12

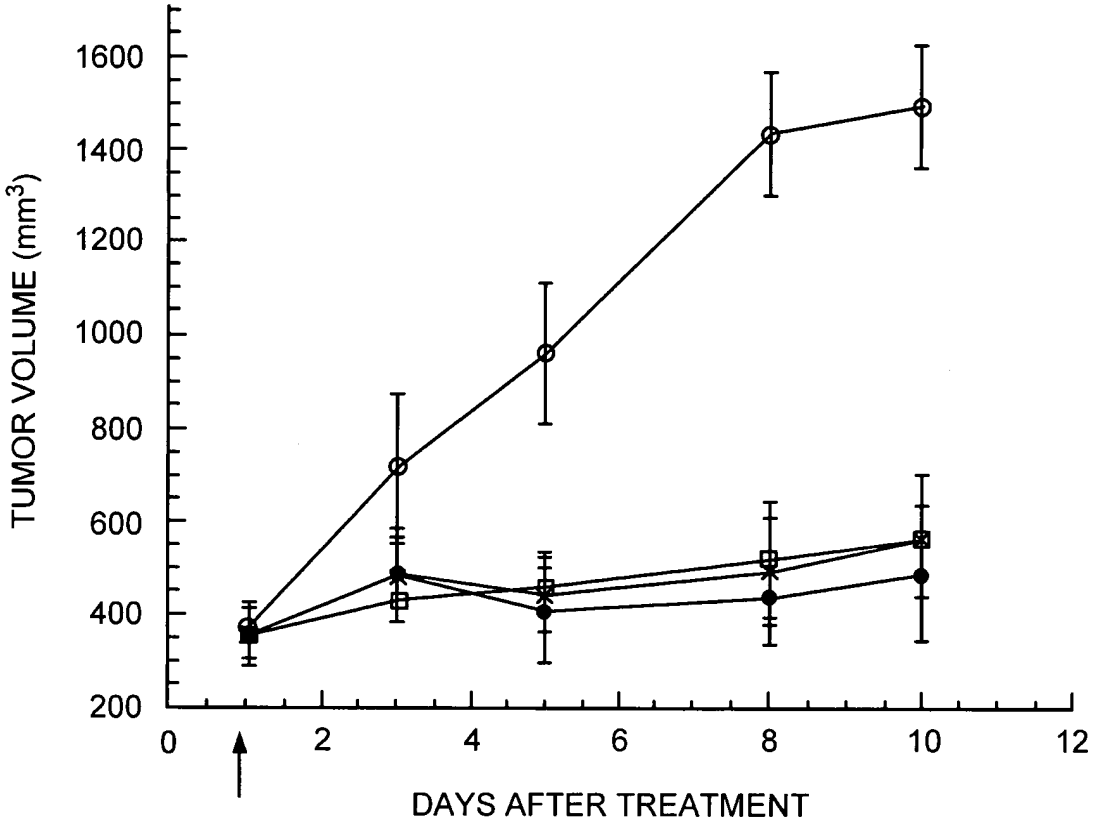


FIG. 13A

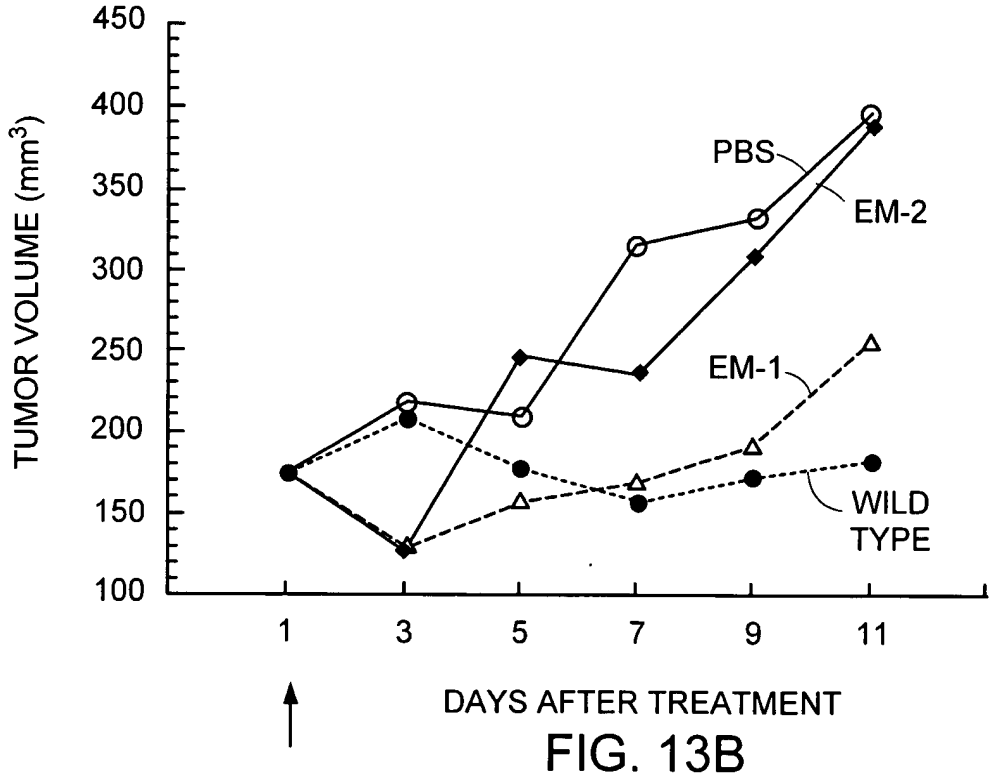


FIG. 13B

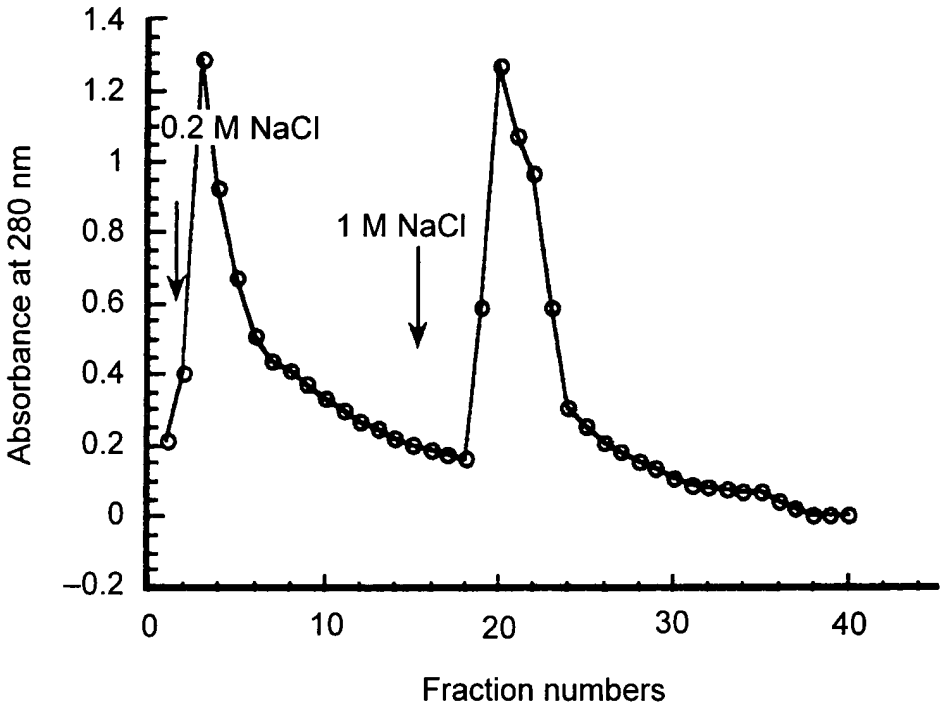


FIG. 14A

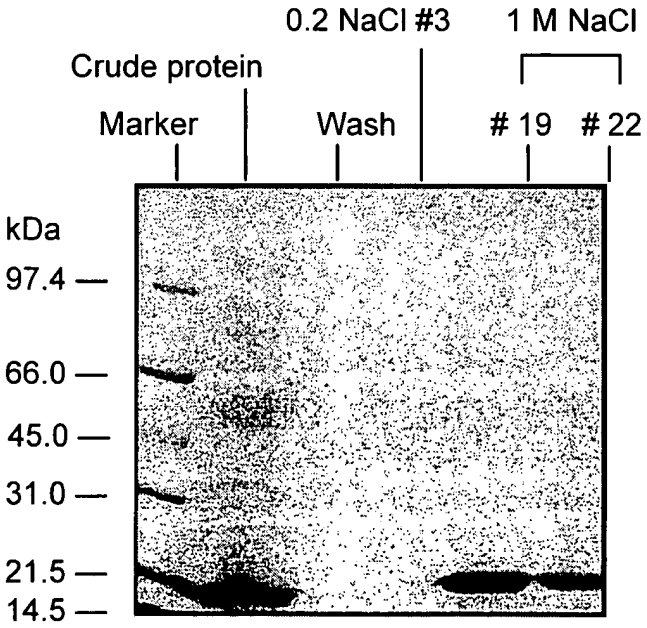


FIG. 14B

HUVE

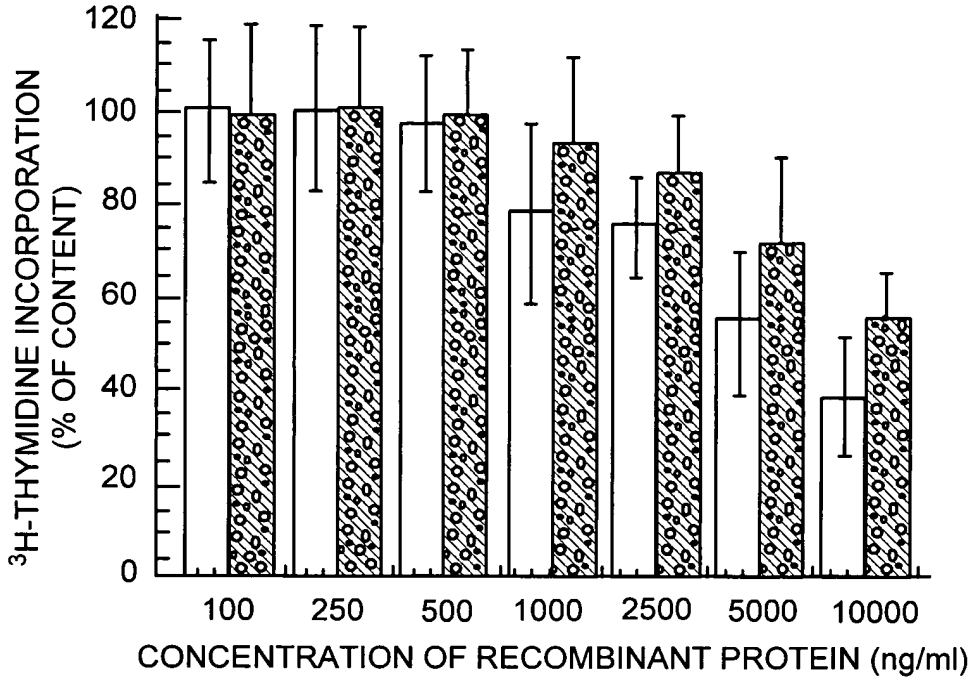


FIG. 15A

HMEV-L

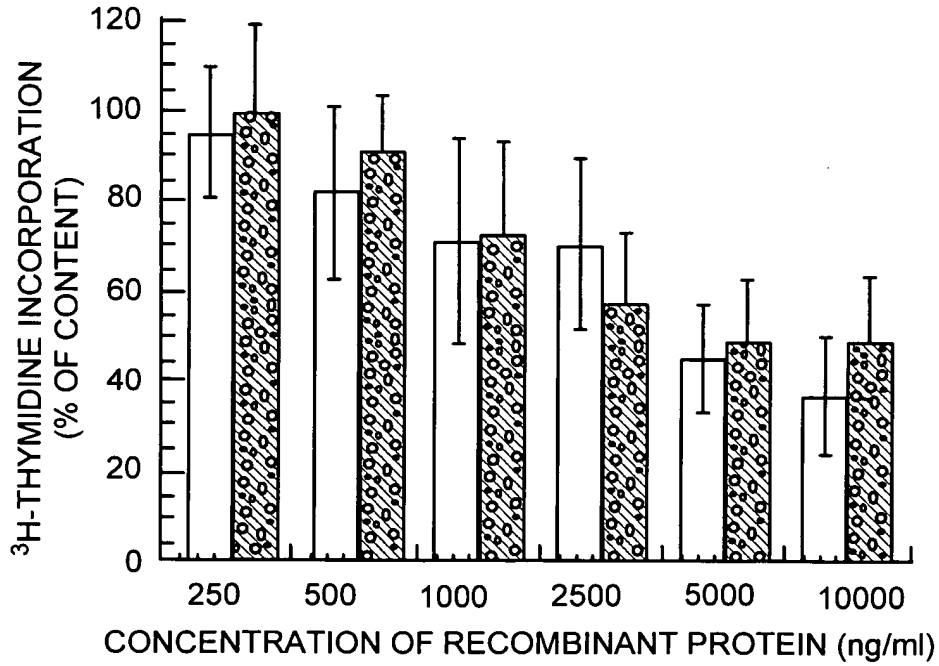


FIG. 15B

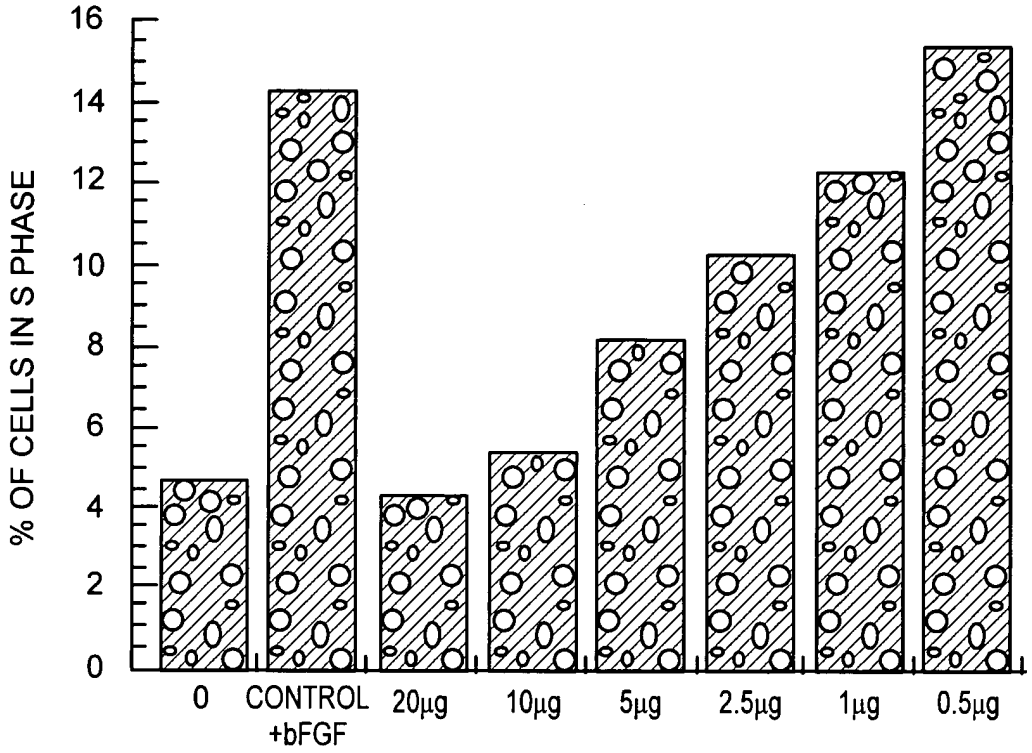


FIG. 16A

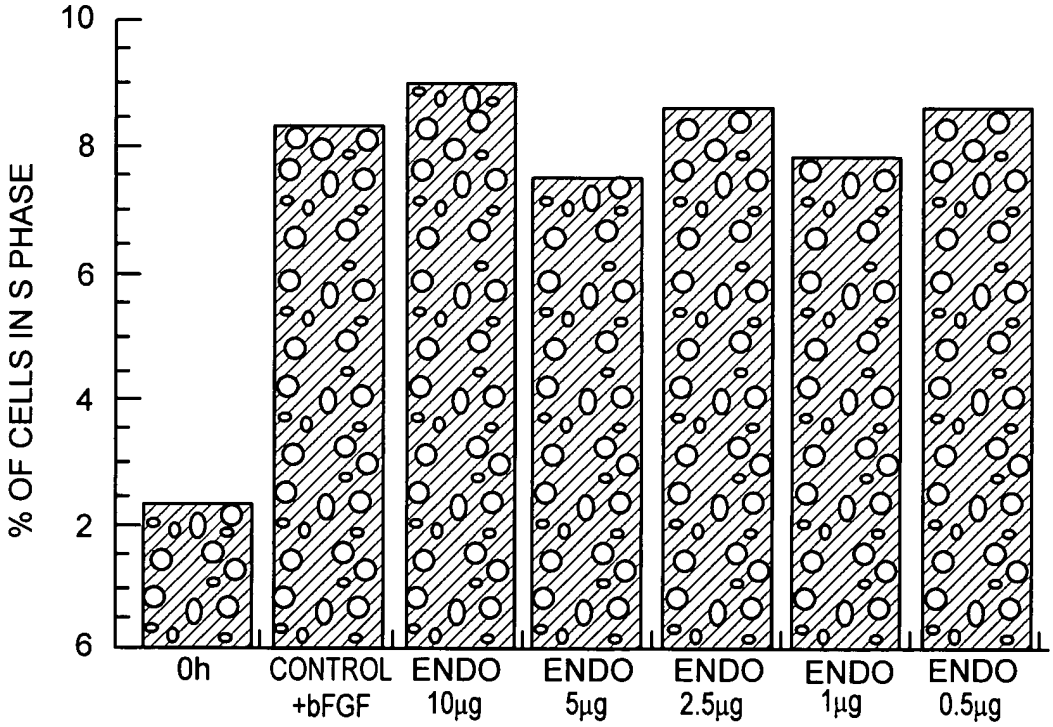


FIG. 16B

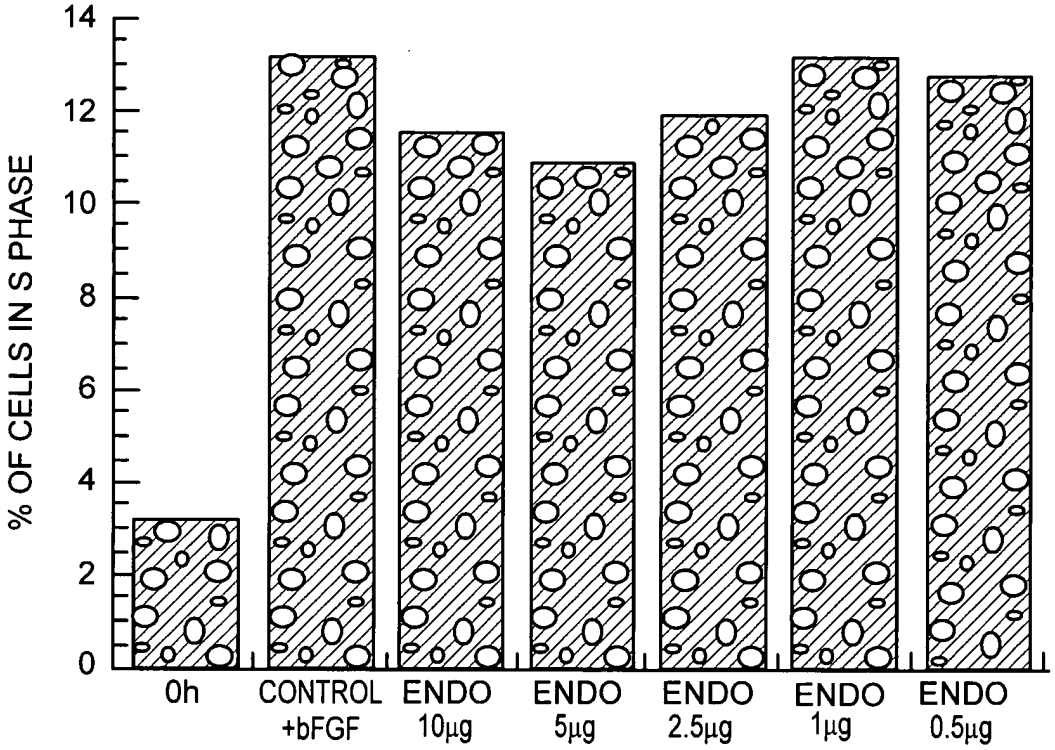


FIG. 16C

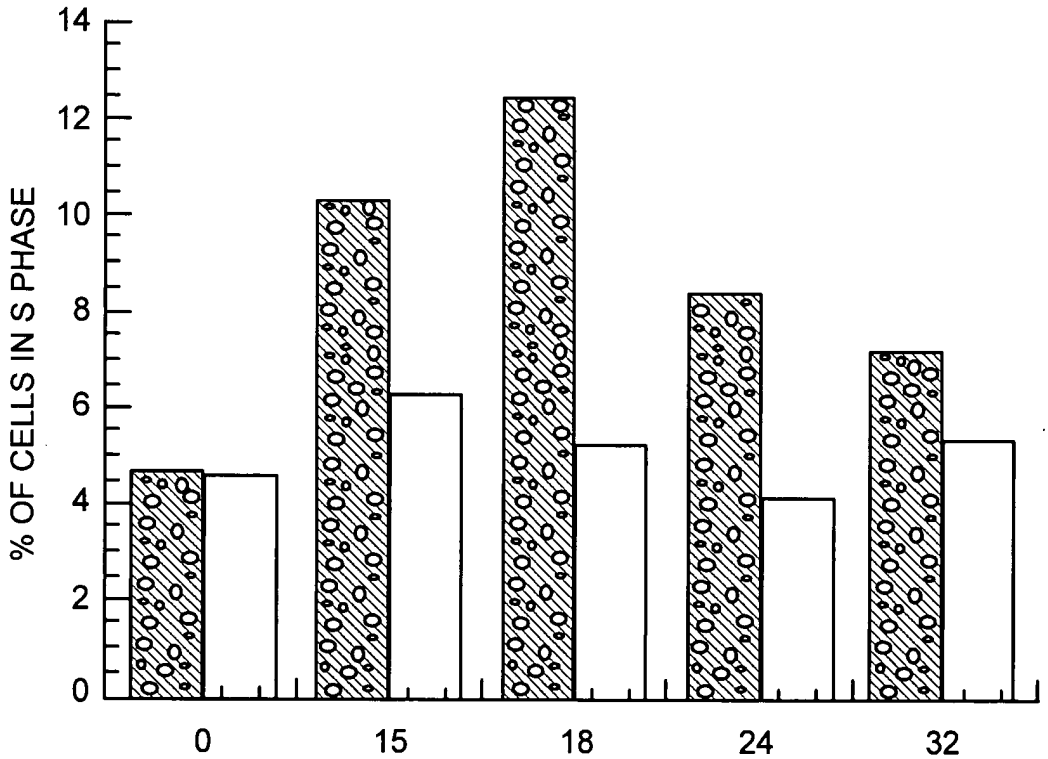


FIG. 16D

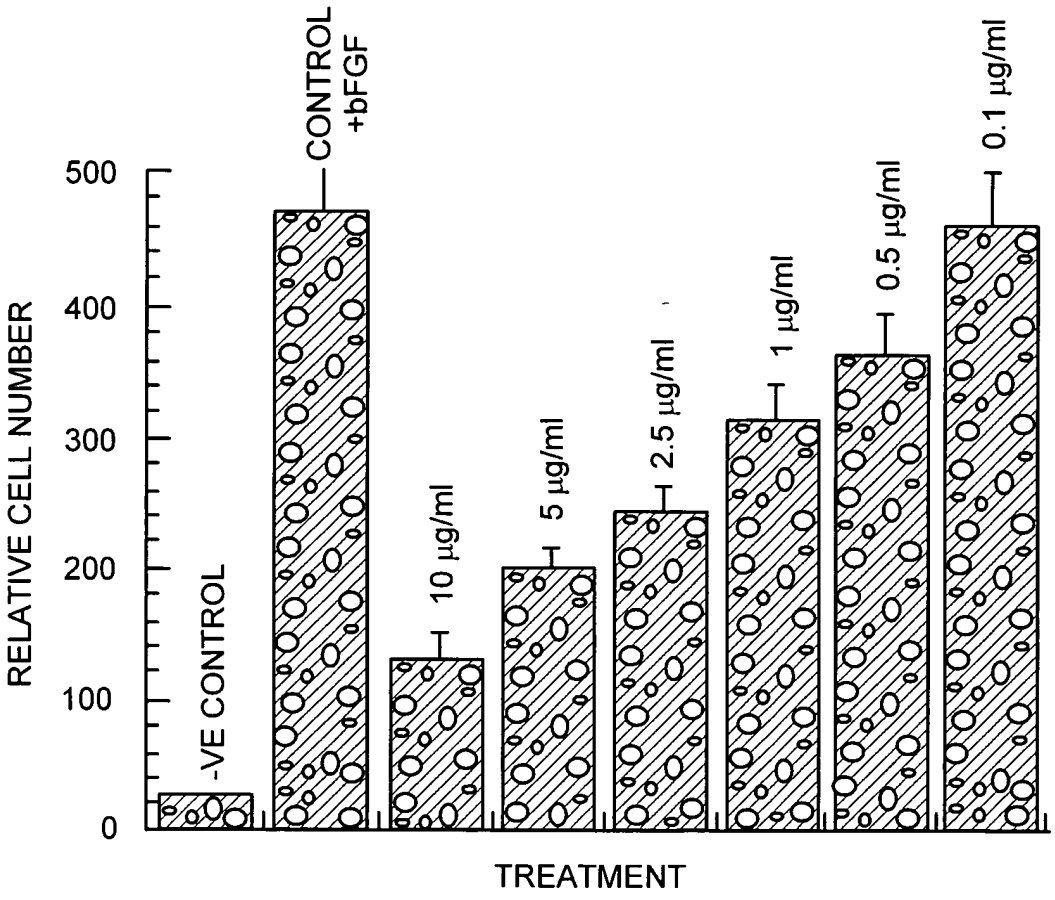


FIG. 17

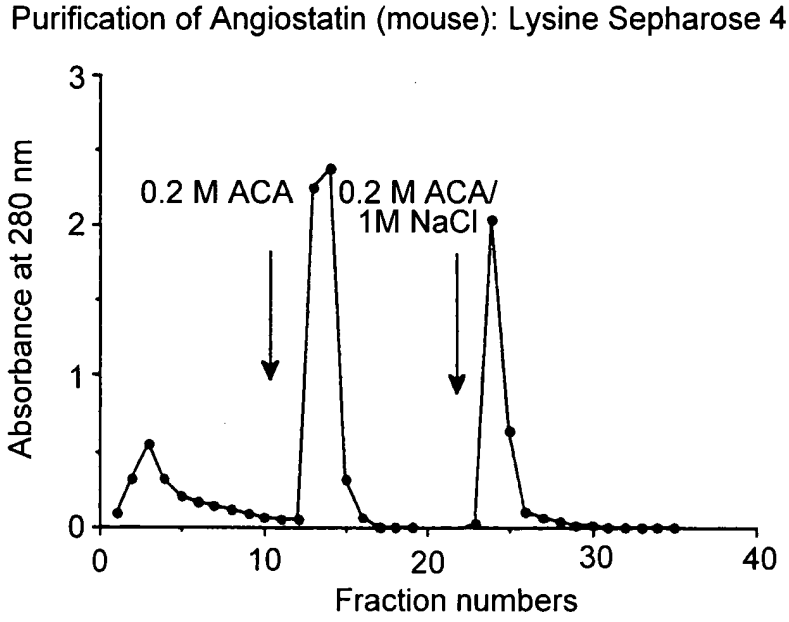


FIG. 18A

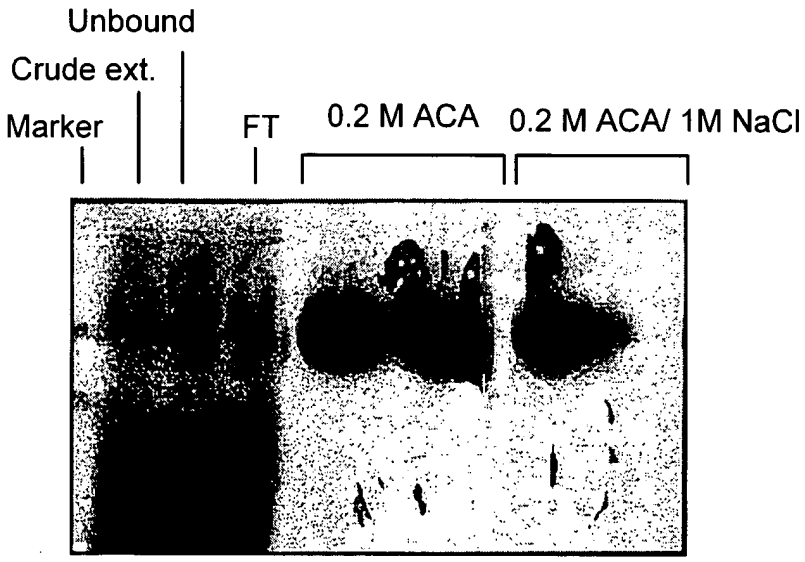


FIG. 18B

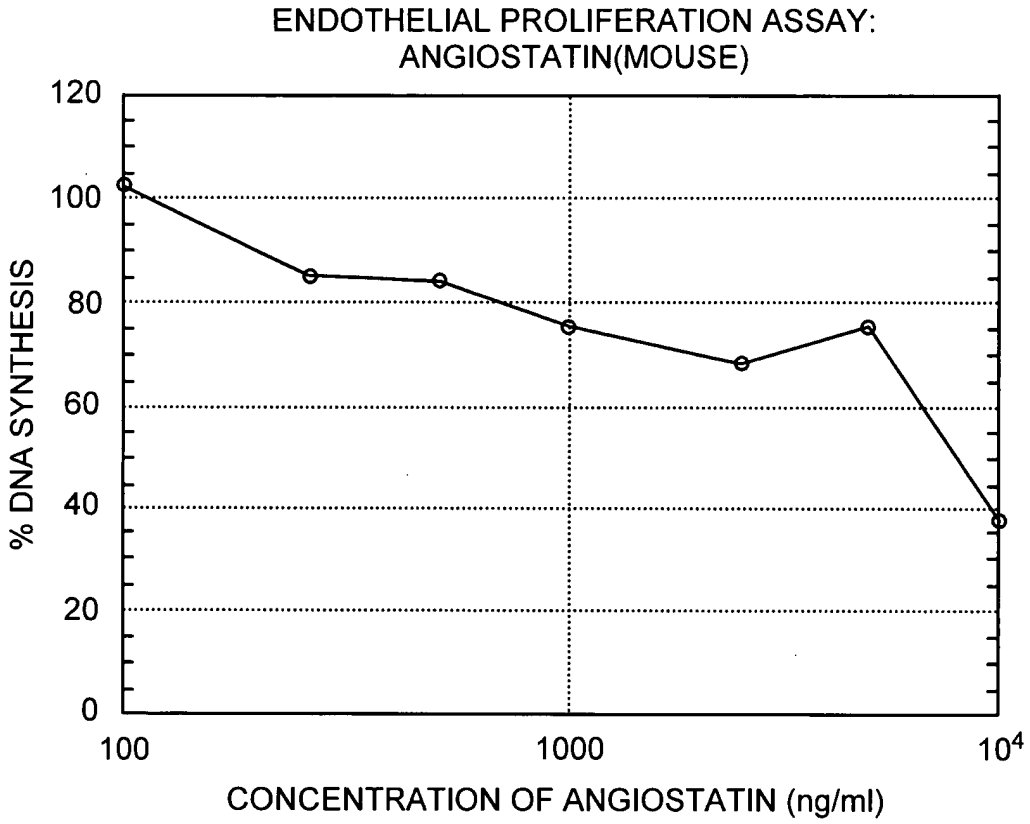


FIG. 19

EFFECT OF RECOMBINANT EDOSTATIN (MOUSE) ON TUMOR GROWTH
(RENAL CANCER CELL LINE 786-0)

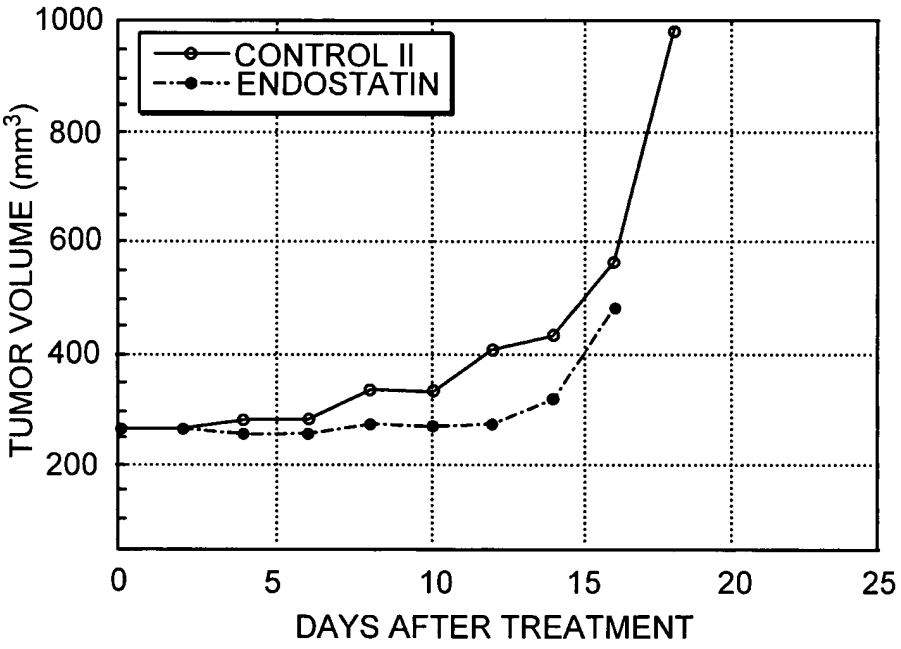


FIG. 20

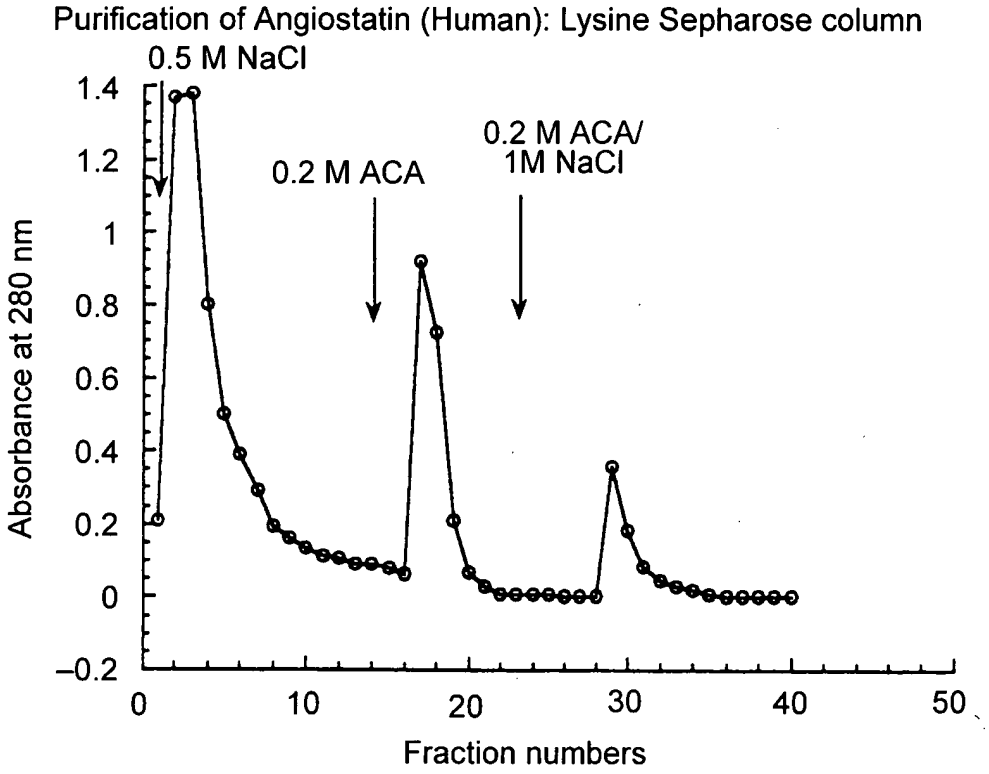


FIG. 21A

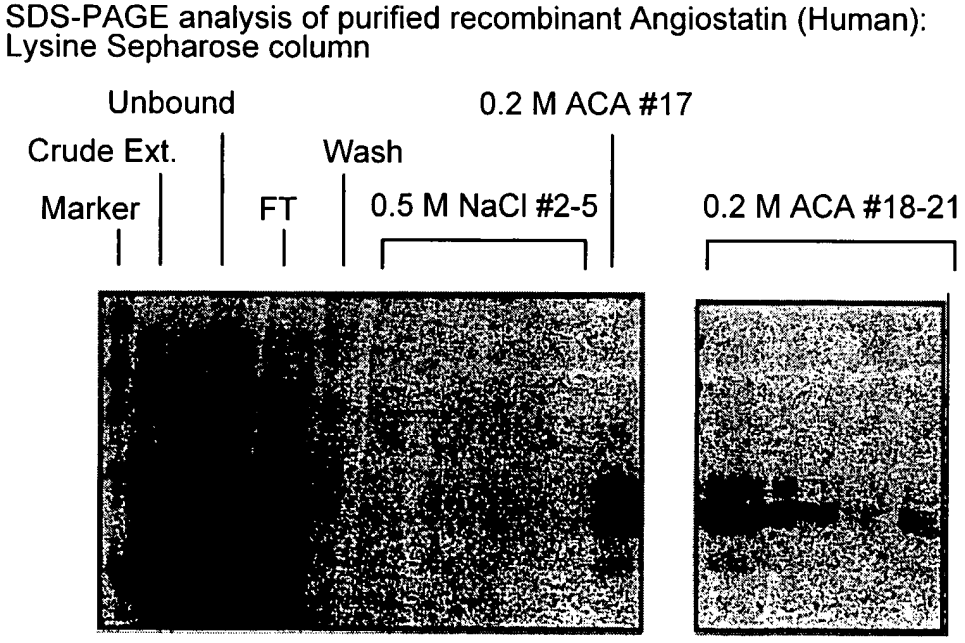


FIG. 21B

FIG. 22A
FIG. 22B

FIG. 22

Sequence Range: 1-546
Upstream primers for Restin and Apomigren are underlined, downstream primer for both is double underlined. Primer nucleotides not in the Restin sequence are shown in lower case.

ttt ttt gaa ttc->

```
      5      10     15      20      25     30      35      40     45
->ATT TCA AGT GCC AAT TAT GAG AAG CCT GCT CTG CAT TG GCT GCT CTG
  TAA AGT TCA CGG TTA ATA CTC TTC GGA CGA GAC GTA AAC CGA CGA GAC

  50      55     60      65      70     75      80      85     90      95
  AAC ATG CCA TTT TCT GGG GAC ATT CGA GCT GAT TTT CAG TGC TTC AAG
  TTG TAC GGT AAA AGA CCC CTG TAA GCT CGA CTA AAA GTC ACG AAG TTC

   100    105     110     115    120     125     130    135     140
 CAG GCC AGA GCT GCA GGA CTG TTG TCC ACC TAC CGA GCA TTC TTA TCT
 GTC CGG TCT CGA CGT CCT GAC AAC AGG TGG ATG GCT CGT AAG AAT AGA

145    150     155     160    165     170     175    180     185     190
  TCC CAT TTG CAA GAT CTG TCC ACC ATT GTG AGG AAA GCA GAG AGA TAC
  AGG GTA AAC GTT CTA GAC AGG TGG TAA CAC TCC TTT CGT CTC TCT ATG

  195     200     205    210     215     220    225     230     235    240
  AGC CTT CCC ATA GTG AAC CTC AAG GGC CAA GTA CTT TTT AAT AAT TGG
  TCG GAA GGG TAT CAC TTG GAG TTC CCG GTT CAT GAA AAA TTA TTA ACC
```

FIG. 22A

```
    245    250    255    260    265    270    275    280    285
  GAC TCA ATT TTT TCT GGC CAC GGA GGT CAG TTC AAT ATG CAT ATT CCA
  CTG AGT TAA AAA AGA CCG GTG CCT CCA GTC AAG TTA TAC GTA TAA GGT

  ttc cat atg->
 290    295    300    305    310    315    320    325    330    335
->ATA TAC TCC TTT GAT GGT CGA GAC ATA ATG ACA GAT CCT TCT TGG CCC
  TAT ATG AGG AAA CTA CCA GCT CTG TAT TAC TGT CTA GGA AGA ACC GGG

   340    345    350    355    360    365    370    375    380
  CAG AAA GTC ATT TGG CAT GGC TCC AGC CCC CAT GGC GTC CGC CTT GTG
  GTC TTT CAG TAA ACC GTA CCG AGG TCG GGG GTA CCG CAG GCG GAA CAC

385    390    395    400    405    410    415    420    425    430
  GAT AAC TAC TGT GAA GCA TGG CGA ACC GCG GAC ACA GCG GTC ACG GGA
  CTA TTG ATG ACA CTT CGT ACC GCT TGG CGC CTG TGT CGC CAG TGC CCT

  435    440    445    450    455    460    465    470    475    480
  CTT GCC TCC CCG CTG AGC ACG GGG AAG ATT CTG GAC CAG AAA GCA TAC
  GAA CGG AGG GGC GAC TCG TGC CCC TTC TAA GAC CTG GTC TTT CGT ATG

    485    490    495    500    505    510    515    520    525
  AGC TGT GCT AAT CGG CTA ATT GTC CTA TGT ATC GAA AAC AGT TTC ATG
  TCG ACA CGA TTA GCC GAT TAA CAG GAT ACA TAG CTT TTG TCA AAG TAC

 530    535    540    545
  ACA GAC GCT AGG AAG TAA
  TGT CTG CGA TCC TTC ATT cgc cgg cgt aag aa
```

FIG. 22B

Sequence Range: 1 to 181

```
     5     10    15     20     25    30     35     40    45
ISS ANY EKP ALH LAA LNM PFS GDI RAD FQC FKQ ARA AGL LST YRA FLS

50     55    60     65     70    75     80     85    90     95
SHL QDL STI VRK AER YSL PIV NLK GQV LFN NWD SIF SGH GGQ FNM HIP

  100   105    110    115   120    125    130   135    140
IYS FDG RDI MTD PSW PQK VIW HGS SPH GVR LVD NYC EAW RTA DTA VTG

145   150    155    160   165    170    175   180
LAS PLS TGK ILD QKA YSC ANR LIV LCI ENS FMT DAR K
```

FIG. 23

FLOW CHART: CLONING OF RESTIN (COIXV) INTO PICHIA EXPRESSION SYSTEM

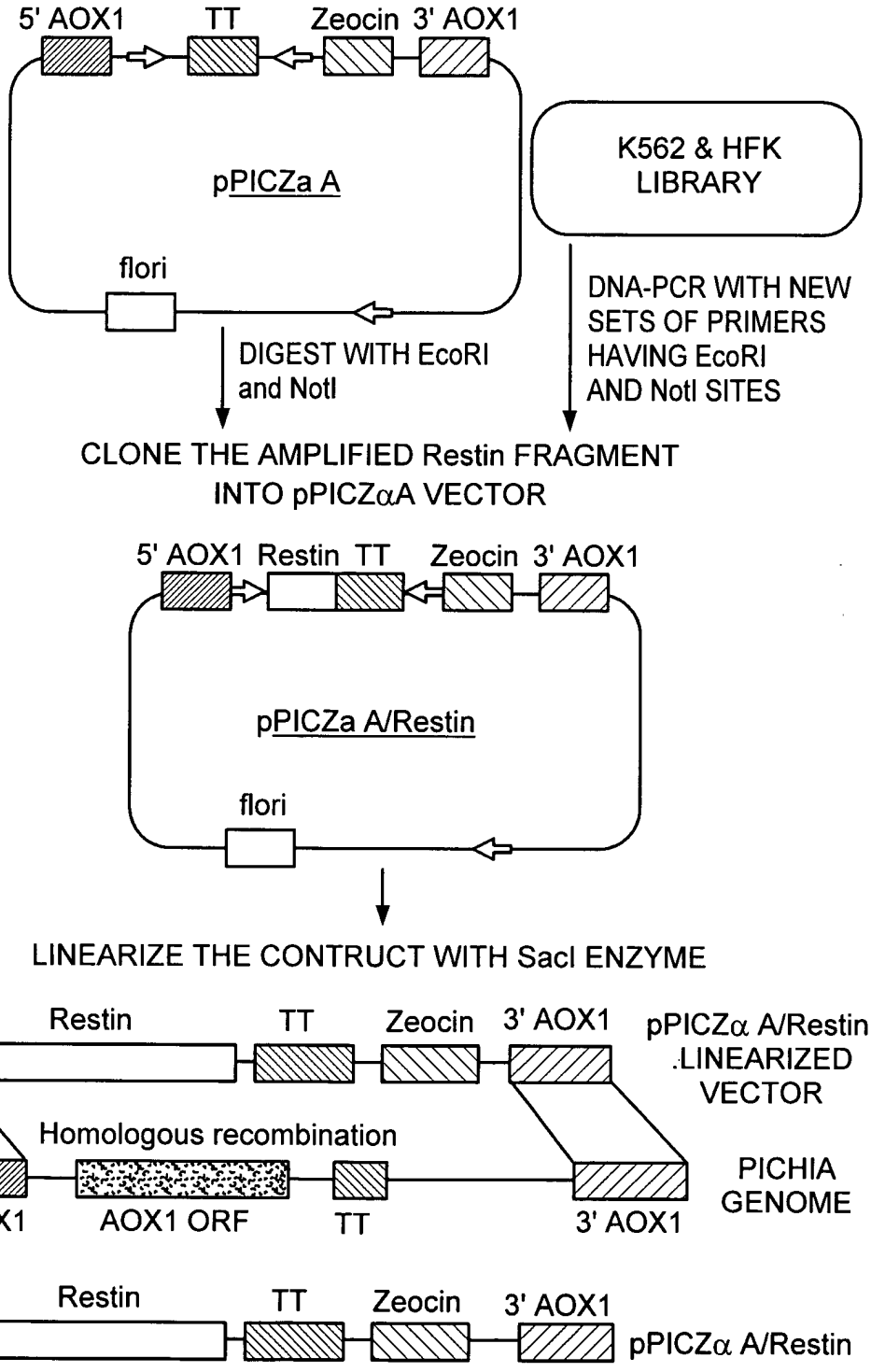


FIG. 24

HUMAN RESTIN

Construct Name	Primer Sequence	Cloning Sites	Vector	Protein Sequence
pPICZαA/ Restin	5' TTT TTT GAA TTC ATT TCA AGT- GCC AAT TAT GAG AAG CCT GCT- CTG CAT-TTG-3' (up) (SEQ ID NO:21) 5' AAG AAT GCG GCC GCT TAC TTC- CTA GCG TCT GTC ATG AAA CTG- TTT TCG AT-3' (down) (SEQ ID NO:22)	*Eco*RI & *Not*I	Eukaryotic (Yeast), Pichia, pPICZαA *(yeast human restin)*	EF-restin (SEQ ID NO:27)
pPICZαA/ His.Restin	5' AAT TCC ATC ACC ATC ACC ATC- ACG-3' (up) (SEQ ID NO:23) 5' AAT TCG TGA TGG TGA TGG TGA-TGG-3' (down) (SEQ ID NO:24)	*Eco*RI (oligo insertion)	Eukaryotic (Yeast), Pichia, pPICZαA *(yeast human his.restin)*	EFHHHHHH-restin (SEQ ID NO:28)
pET28a/ apomigren	5' TTC CAT ATG ATA TAC TCC TTT- GAT GGT CGA GAC ATA ATG ACA-3' (up) (SEQ ID NO:25) 5' AAT GCG GCC GCT TAC TTC CTA- GCG TCT GTC ATG AAA CTG TTT- TCG AT-3' (down) (SEQ ID NO:26)	*Nde*I & *Not*I	Prokaryotic, pET system *(E.coli human apomigren)*	MGSSHHHHHHSSGLVPRGSHM-apomigren (SEQ ID NO:29)
pPICZαA/ apomigren	5' AAG AAT TCC ATC ATC ATC ATC- ATC ACA GCA GC-3' (up) (SEQ ID NO:11) 5' AAT GCG GCC GCT TAC TTC CTA- GCG TCT GTC ATG AAA CTG TTT- TCG AT-3' (down) (SEQ ID NO:26)	*Eco*RI & *Not*I	Eukaryotic (Yeast), Pichia, pPICZαA *(yeast human apomigren)*	EFMGSSHHHHHHSSGLVPRGSHM-apomigren (SEQ ID NO:30)

FIG. 25

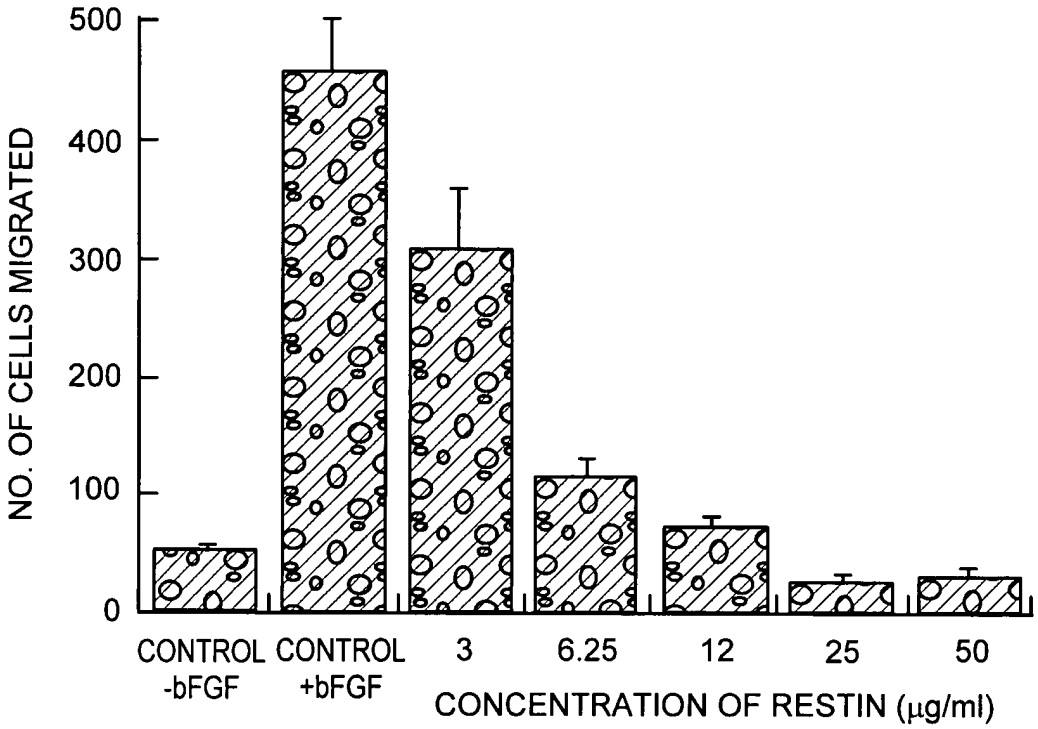


FIG. 26

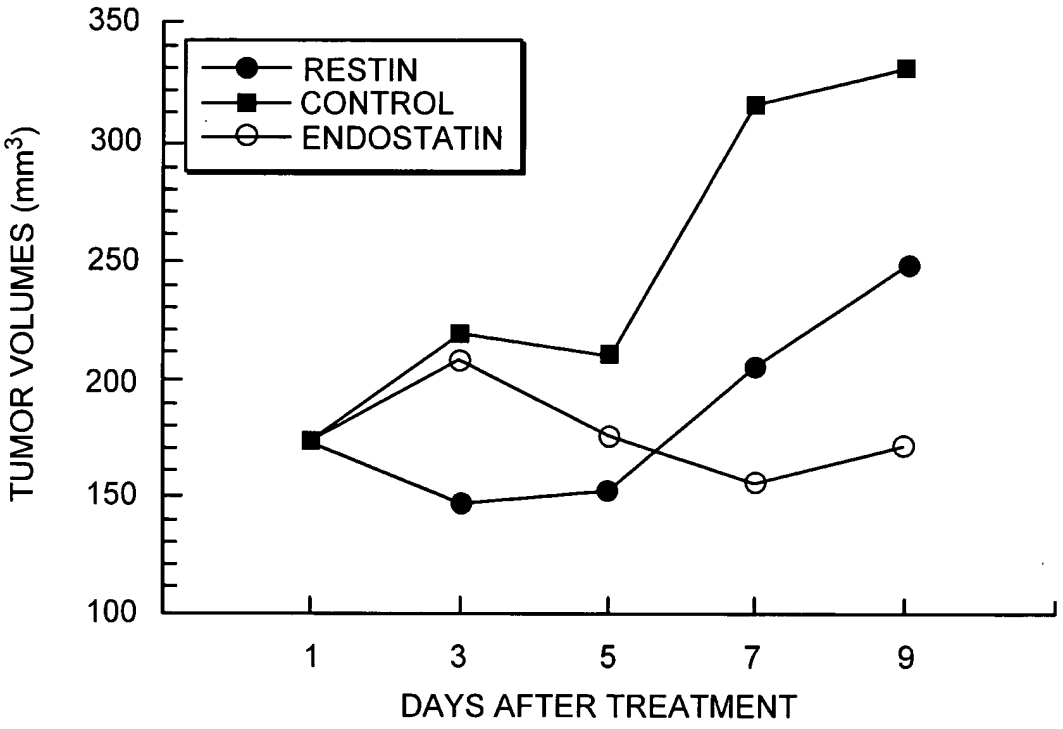


FIG. 27